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ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EFFECT OF NITRIC OXIDE SYNTHASE ON BETA 3 ADRENOCEPTOR MEDIATED RELAXATION IN STZ DIABETIC RAT HEART

STZ DİABETİK SIÇAN KALBİNDE BETA 3 ADRENOSEPTÖR ARACILI GEVŞEME YANITINDA NİTRİK OKSİT SENTAZIN ETKİSİ

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ABSTRACT

Objective: *Beta 3 adrenoceptors have been shown to mediate negative inotropic effect in the heart. In the dibetic rat heart, beta 3 adrenoceptor related relaxation response has been found to be augmented. In the present study, we aimed to investigate the contribution of nitric oxide and nitric oxide synthase subtypes on beta 3 adrenoceptor mediated relaxation response in chronic STZ diabetic rat heart.*

Material and Method: *8-week old sprague dawley rats were used in the study. Diabetes was induced by single dose streptozotocin injection. Some of the diabetic rats were treated with insulin. Beta 3 adrenoceptor mediated negative inotropic effect was evaluated by using CL 316,243 in the absence and presence of L-NAME. The expression of beta 3 adrenoceptor, eNOS, phosphorylated eNOS and nNOS were determined by western blot experiments.*

Result and Discussion: *The relaxation response was increased in diabetic heart and not normalized after insulin treatment. The effect was abolished after L-NAME incubation. Beta 3 adrenoceptor was upregulated in diabetic heart and reached to control values after treatment. Expression of eNOS, phosphorylated eNOS or nNOS was not altered significantly among the groups. The results of the study indicate that beta 3 adrenoceptor mediated relaxation in diabetic heart is nitric oxide related.*

Keywords: *Diabetes, heart, beta 3 adrenoceptor, nitric oxide synthase*

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ÖZ

Amaç: *Beta 3 adrenoseptörlerin kalpte negatif inotropik etkiye aracılık ettiği gösterilmiştir. Diabetik sıçan kalbinde, beta 3 adrenoseptör aracılı gevşeme yanıtının belirgin hale geldiği bulunmuştur. Bu çalışmada, kronik STZ diabetik sıçan kalbinde nitrik oksit ve nitrik oksit sentetaz alttiplerinin beta 3 adrenoseptör aracılı gevşeme yanıtına katkısının araştırılması amaçlanmıştır.*

Gereç ve Yöntem: *Çalışmada 8-haftalık sprague dawley sıçan kullanılmıştır. Diabet tek doz streptozotosin injeksiyonu ile gerçekleştirilmiştir. Bazı diabetik sıçanlar insulin ile tedavi edilmiştir. Beta 3 adrenoseptör aracılı gevşeme yanıtı CL 316,243 kullanılarak L-NAME varlığında ve yokluğunda değerlendirilmiştir. Beta 3 adrenoseptör, eNOS, fosforile eNOS ve nNOS ekspresyonu western blot yöntemi ile ölçülmüştür.*

Sonuç ve Tartışma: *Diabetik kalpte CL-aracılı gevşeme yanıtları artmıştır, ancak insulin tedavisi sonrasında kontrol düzeyine gelmemiştir. Gözlenen etki L-NAME inkübasyonu sonrasında ortadan kalkmıştır. Beta 3 adrenoseptör ekspresyonu diabetik kalpte artmıştır ve tedavi sonrası kontrol seviyesine geri dönmüştür. eNOS, fosforile eNOS ve nNOS düzeyleri gruplararasında anlamlı bir fark bulunamamıştır. Çalışmanın sonuçları, diabetik sıçan kalbinde beta 3 adrenoseptör aracılı gevşeme yanıtının nitrik oksit bağlantılı olduğunu ortaya koymaktadır.*

Anahtar Kelimeler: *Diabet, kalp, beta 3 adrenoseptör, nitrik oksit sentaz*

INTRODUCTION

Beta 3 adrenoceptors (AR), the third subtype of beta ARs, have been first demonstrated in adipose tissue where they mediate thermogenesis and lipolysis [1]. The presence of this subtype in cardiac tissue has been shown by Gauthier et al. [2]. In human endomyocardial biopsies, beta 3 ARs have been found to mediate negative inotropic effect. This effect has been suggested to be mediated through nitric oxide (NO)-cyclic guanosine mono phosphate (cGMP)-protein kinase G (PKG) [3]. An important feature of beta 3 ARs is that this subtype exert interspecies differences [4] which means it is difficult to extrapolate the findings on animals to the ones in humans.

Beta 3 ARs have been thought not to be functional in the healthy heart. This subtype has been suggested to have a role in cardiac pathologies with excessive catecholamine drive such as heart failure [5] or diabetes [6]. Beta 3 AR mediated negative inotpic effect has been interpreted as an adaptive mechanism to protect the heart from the deleterious effects of sympathetic nervous system overactivation [7]. From that point of view, there are some ongoing clinical studies which investigate the possible beneficial effects of beta 3 AR agonist Mirabegron in heart failure [8].

In diabetic rat heart, beta 3 ARs have been demonstrated to be upregulated [6]. This subtype has been reported to cause negative inotropy in Langendorff perfused heart of STZ diabetic rats [9]. Nitric oxide synthase (NOS) has been suggested to have role in beta 3 AR mediated effect [7]. However, beta 3 AR mediated relaxation response has not been studied in chronic diabetic heart in terms of NOS. Furthermore, it is not clear which NOS subtype is responsible for that response. Thus, in the present study, we aimed to investigate the effect of NOS on beta 3 AR mediated relaxation response in chronic STZ diabetic rat heart.

MATERIAL AND METHOD

Animals:

10-week old male Sprague Dawley rats (200–250 g) were obtained from Bilkent University (Ankara, Turkey). The rats were housed individually on a 12 h light/dark cycle at constant room temperature. The rats were fed with standard laboratory chow (Purina Rat Chow; Optima AS, Turkey) and had tap water ad libitum in the Ankara University Faculty of Pharmacy Animal Care Unit. All experiments were approved by the Ankara University Animal Care and Use Committee (2010-56-283).

Induction of experimental diabetes:

Diabetes was induced with single dose of streptozotocin (STZ, 40 mg/kg ,i.p., Sigma-Aldrich) dissolved in citrate buffer (pH 4.5). After 72 hours, blood glucose was measured and the rats with a glucose value higher than 300 mg/dl were accepted as diabetic.

Insulin treatment protocol:

After twelve weeks of STZ injection, diabetic rats were randomly divided as diabetic and insulin treated diabetic. Treated rats were injected twice daily with neutral protamine Hagedorn (NPH) insulin (10-18 U/kg/day, s.c., Humulin® N, Eli Lilly, USA) for two weeks. Blood glucose levels were measured before each injection using Accu-check® (Roche Diagnostics) strips.

Langendorff heart preparation:

Beta 3-AR-mediated relaxation response was determined by using Langendorff perfused hearts. Rats were anaesthetized with ketamin (60 mg/kg) and xylazine (10 mg/kg) combination (i.p.). the heart were excised and perfused retrogradely with modified Krebs-Henseleit solution (mmol/L; 120 NaCl, 4.8 KCl, 1.25 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose; 37°C, pH= 7.4). Coronary perfusion was made at a rate of 10 ml/min. Hearts were paced at 300 bpm. A latex balloon was inserted to the left ventricle. Beta 3 AR mediated relaxation was evaluated by using CL 316, 243 (Sigma, USA), a selective beta 3 AR agonist (0,1nM-10 µM). The experiment was repeated in the presence of L-NAME (Sigma, USA), a nonselective NOS inhibitor (0,001 µM) to determine the effect of NOS in beta 3 AR mediated relaxation.

Western blot experiments:

Left ventricle tissues were homogenized with RIPA buffer (Sigma, USA) and sonicated. Then, cardiac homogenates were centrifuged at 16,000g for 30 min at $+4^0$C. Protein concentrations were determined by using the BCA assay (Pierce, Thermo, USA). Protein samples (100 ug per lane) were separated on SDS PAGE gels (6-10%) and transferred to PVDF membrane (Bio-Rad, USA). After blocking with 5% BSA, membranes were incubated with primary antibody overnight at $+4^0$C (β3-adrenoceptor (1:500, Abcam, USA); eNOS (1:500, Cell signaling, USA); phophorylated eNOS (1:500, Cell signaling, USA); nNOS (1:500, Cell signaling, USA); α-tubulin (1:10.000, Abcam, USA). Then membranes were washed with TBST and incubated with secondary antibody for 2 h at $+4^0$C (antirabbit

(1:2000, Cell signaling, USA), antirabbit (1:5000, Abcam, USA), antichicken (1:2000, AnaSpec, USA). After washing with TBST, blots were detected by enhanced chemiluminescense assay (Pierce ECL, Thermo, USA). Bands were quantified by using Image J (NIH, USA). Densitometric values were normalized to control gene α-tubulin.

Statistical analysis:

The results are expressed as mean±S.E.M. Statistical significance was tested by ANOVA followed by Bonferroni's post hoc test for multiple comparisons of group means. The analysis was performed using the statistical software package (Graphpad Prism, USA). The probability level of $P<0.05$ was considered as statistical significance.

RESULT AND DISCUSSION

In the present study, we evaluated beta 3 AR mediated relaxation response in STZ diabetic rats. At the end of the 16-week diabetes period, the blood glucose level was significantly higher in diabetic group as expected (table 1). 2-week insulin treatment normalized these values.Body weight was decreased in diabetic group and insulin treatment improved it (table 1). Heart weight was decreased in diabetic group and reached to control value after insulin treatment. On the other hand, heart weight to body weight ratio was increased in diabetic group and was not changed after treatment (Table 1).

Table 1. General characteristics of rats.

	C	**D**	**DI**
BG	116,6±2,81	446,4±10,86***	90,9±5,05*###
BW	351±11,63	298,36±9,50**	341,4±7,67##
HW	1,44±0,02	1,24±0,03**	1,44±0,04##
HW/BW	0,0038±0,0001	0,0045±0,0002*	0,0043±0,0001

C, Control; D, Diabetic; DI, Insulin treated diabetic. BG, blood glucose; BW, body weight; HW, heart weight.*, $p<0.005$, **, $p<0.01$, ***, $p<0.001$, C vs D; ##, $p<0.01$, ###,$p<0.001$, D vs DI.

Our results indicate that beta 3 AR mediated relaxation response was augmented in chronic STZ diabetes and this response is NOS dependent. CL 316, 243, a selective beta 3 AR agonist caused negative inotropy in rat heart and this response was increased in chronic diabetes (figure 1, 2). Beta 3 ARs have been reported to cause negative inotropy in Langendorff perfused heart of STZ diabetic rats [9]. On the other hand, it has been found that relaxation response mediated by beta 3 ARs was blunted in the cardiac papillary muscle of STZ diabetic rats [10]. This difference has been suggested to result from the experimental approach. As is known, for Langendorff heart preparation whole heart is used which means coronary arteries, atria and ventricles are involved in this response. However, papillary muscle is a tissue

excised from left ventricle which makes the response more spesific to left ventricle. One could assume that relaxation of coronary arteries by CL 316, 243 could have attributed to the response obtained in the Langendorff perfused heart.

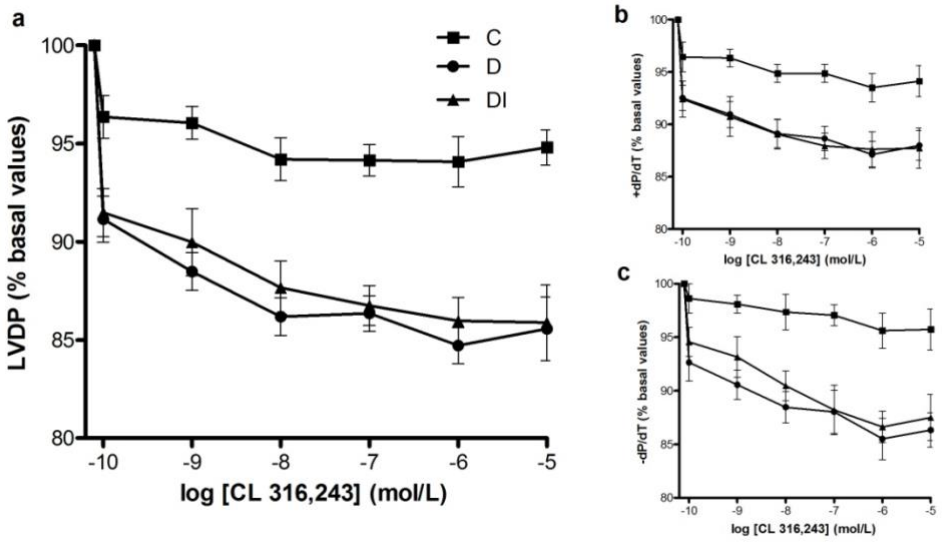

Figure 1. CL 316,243 mediated relaxation response in Langendorff perfused rat heart. LVDP, left ventricle developed pressure; $^{+/-}$ dp/dt, rate of contraction and relaxation. C, control, D, diabetic, DI, insulin treated diabetic.

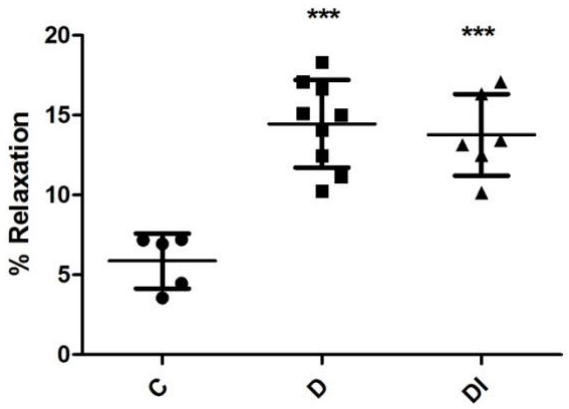

Figure 2. Emax values for CL 316,243 mediated relaxation response at the dose of 1µM. C, control, D, diabetic, DI, insulin treated diabetic. ***, $p<0.001$, C vs D.

The results of the present study are consistent with our previous findings as we have found that BRL 37341, a preferential beta 3 AR agonist, resulted in increased negative inotropic effect in 8-week

STZ diabetic rat heart [9]. We evaluated the response also in the presence of NOS inhibitor. We observed that negative inotropic effect of CL 316,243 was abolished after incubation with L-NAME (figure 3). However, it is not clear which subtype of NOS is responsible for this effect. Unfortunately, we could not have determined the possible alteration in the response in the presence of selective nNOS inhibitor. Using subtype spesific NOS inhibitors could help to clarify the mechanism of beta 3 AR mediated negative inotropic effect in chronic diabetes.

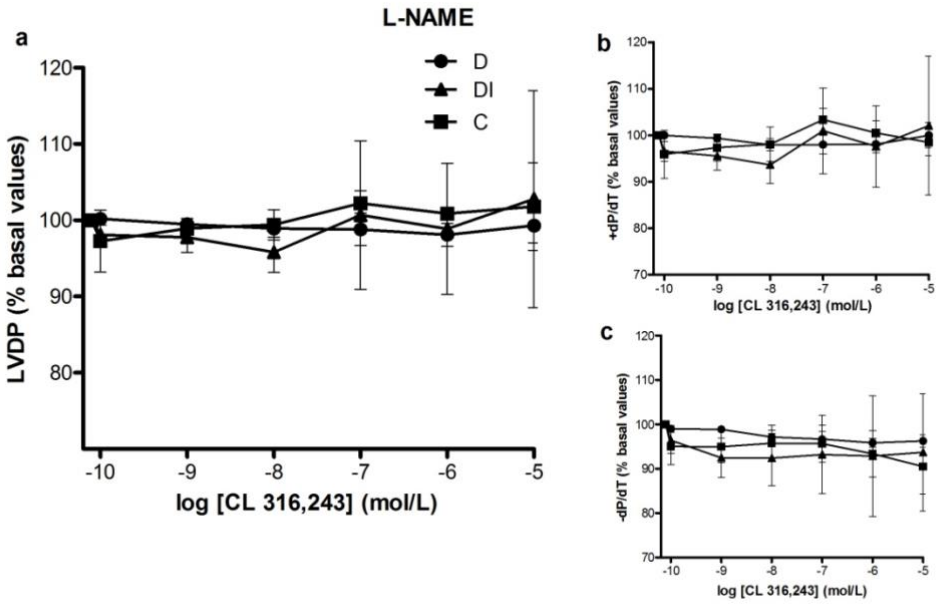

Figure 3. CL 316,243 mediated relaxation response in the presence of L-NAME (0,01µM) in the Langendorff perfused heart. LVDP, left ventricle developed pressure; $^{+/-}$ dp/dt, rate of contraction and relaxation. C, control, D, diabetic, DI, insulin treated diabetic.

We have shown that protein expression of beta 3 AR was upregulated in diabetic rat heart (figure 4). This finding is line with previous results as beta 3 AR upregulation has been previously shown in diabetes [6]. In addition, we have also determined eNOS and nNOS expression which were not significantly changed in diabetes (figure 4). This data is not consistent with the results of Amour et al [7] since they found that expression of eNOS is decreased whereas nNOS is upregulated in diabetic rat heart. Actually, in our study, expression of eNOS was decreased to some extent in diabetic heart, however, that was statistically insignificant. eNOS activation was also not changed in diabetes or after treatment as the ratio of phosphorylated eNOS to eNOS did not differ significantly (figure 4). On the other hand, we did not observe any alteration in the expression of nNOS in contrast to the results of Amour et al [7]. The difference could be resulted from the duration of diabetes. In the study of Amour et al [7], they used 4-week diabetic rats which does not refer to chronic diabetes. On the other hand, in

our study the duration was 16 weeks rats which could be regarded as chronic diabetes. Thus, it could be assumed that the expression of NOS subtypes could be changed as the disease progresses.

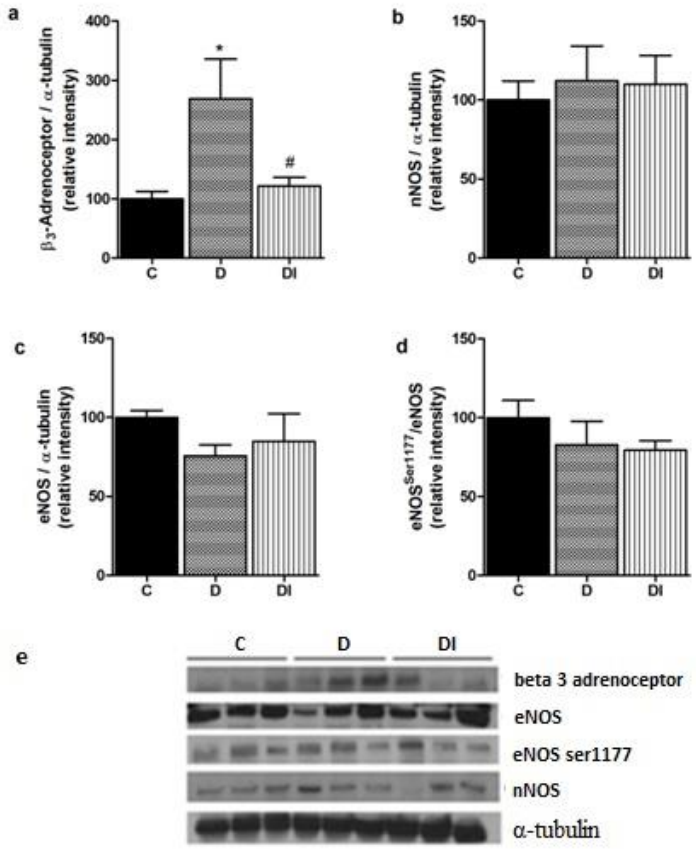

Figure 4. Protein expression bar graphs and representive protein bands for each group. C, control, D, diabetic, DI, insulin treated diabetic. *, $p<0.05$, C vs D; #, $p<0.05$, D vs DI.

Another interesting point is that insulin treatment did not change the expression of the NOS subtypes. It only normalized beta 3 AR expression which was not correlated with the increased relaxation response in the treated group. The reason for augmented relaxation response in treated group despite of the normalized expression of the receptor could be attributed to the possible alterations in the beta 3 AR signaling pathway. In this regard, eNOS or NOS should be excluded as no significant change was observed. However, some other factors such as NO bioavailibility, the changes in cGMP or PKG could have a role. This should be further clarified.

In the conclusion, our present findings indicate that beta 3 AR mediated negative inotropic effect is NOS dependent in chronic STZ diabetic rat heart. However, the contribution of NOS subtypes to this response remains to be elucidated.

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REFERENCES

1. Harms, H.H., Zaagsma, J., van der Wal, B. (1974). Beta-adrenoceptor studies. III. on the beta-adrenoceptors in rat adipose tissue. European Journal of Pharmacology, 25**,** 87-97.
2. Gauthier, C., Tavernier, G., Charpentier, F., Langin, D, Le Marec, H. (1996). Functional beta 3 adrenoceptor in the human heart. Journal of Clinical Investigation, 98, 556–562.
3. Gauthier, C., Leblais, V., Kobzik, L., Trochu, J.N., Khandoudi, N., Bril, A., Balligand, J. L., Le Marec, H. (1998). The negative inotropic effect of beta3-adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. The Journal of Clinical Investigation, 102**,** 1377-1384.
4. Gauthier, C., Tavernier, G., Trochu, J.N., Leblais, V., Laurent, K., Langin, D., Escande, D., Le Marec, L. (1999). Interspecies differences in the cardiac negative inotropic effects of ß$_3$-adrenoceptor agonists. Journal of Pharmacology Experimental Therapeutics, 290**,** 687-693.
5. Moniotte, S., Kobzik, L., Feron, O., Trochu, J.N., Gauthier, C., Balligand, J.L. (2001). Upregulation of ß$_3$-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. Circulation, 103**,** 1649-1655.
6. Dinçer, Ü.D., Bidasee, K.R., Güner, Ş., Tay, A., Özçelikay, A.T., Altan, V.M. (2001). The effect of diabetes on expression of β_1-, β_2-, and β_3-adrenoreceptors in rat hearts. Diabetes, 50**,** 455-461.
7. Amour, J., Loyer, X., Le Guen, M., Mabrouk, N., David, J.S., Camors, E., Carusio, N., Vivien, B., Andriantsitohaina, R., Heymes, C., Riou, B. (2007). Altered contractile response due to increased β_3-adrenoceptor stimulation in diabetic cardiomyopathy. The role of nitric oxide synthase 1–derived nitric oxide. Anesthesiology, 107**,** 452-460.
8. Farah, C., Michel, L.Y.M., Balligand, J.L. (2018). Nitric oxide signalling in cardiovascular health and disease. Nature Reviews Cardiology, 15**,** 292.
9. Kayki-Mutlu, G., Arioglu-Inan, E., Ozakca, I., Ozcelikay, A.T., Altan, V.M. (2014). b$_3$-Adrenoceptor-mediated responses in diabetic rat heart. General Physiology Biophysics, 33**,** 99-109.
10. Arioglu, E., Guner, S., Ozakca, I., Altan, V.M., Ozcelikay, A.T. (2010). The changes in β-adrenoceptor-mediated cardiac function in experimental hypothyroidism: the possible contribution of cardiac β3-adrenoceptors. Molecular and Cellular Biochemistry, 335**,** 59-66.